



SUBSTITUTE SPECIFICATION (CLEAN VERSION)

Serial No. 09/463,801

1374-4 PCT/US

Means for Producing and/or Treating Alcoholic Beverages, Especially Wine or Sparkling Wine, as Well as Its Applications

BACKGROUND OF THE INVENTION

Field of the Invention

The invention refers to means for producing and/or treating alcoholic beverages, especially wine or sparkling wine, in accordance with the preamble of the main claim as well as its applications.

5 Description of the Prior Art

Various species of microorganisms, especially yeast used for alcohol fermentation, are employed in the production of alcoholic beverages, especially of wine or sparkling wine. To optimize the results, other species of microorganisms and enzymes are added to the product or its preliminary stages. Thus, for example, lactic acid bacteria serve to break down malic acid and
10 pectinases accelerate must clarification.

The process of alcohol fermentation can be interrupted by such processes as rapid cooling down, addition of sulfur dioxide, or filtration. These processes for inactivating the yeast are, however, costly, only imprecisely controllable, and can impair the product's quality. Thus, a time-delayed use of various yeast species turns out to be costly.

15 After handling, for example, a wine or lactic acid bacteria to reduce the acid content, the added microorganisms have to be separated, which takes place with the relatively small lactic acid bacteria via membrane filtration. However, in this connection there is not always a guarantee of complete removal of the microorganisms. Lactic acid bacteria remaining in the wine can transform glucose into acetic acid, thus ruining the wine.

20 Enzymes such as proteases to break down peptides and proteins are added to the product or its preliminary stages in liquid form. The inactivation of enzymes usually takes place by heating, whereby impairment of the product can result, as well as a recycling of in part expensive enzymes, is excluded.

There are means known in sparkling wine production for bottle fermentation that consist of yeast immobilized in alginate beads (G. Troost, et al., Sekt, Schaumwein, Perlwein [Champagne, Sparkling Wine, Perlant Wine] Stuttgart 1995 and DE 39 08 997 A1). With this, the time-consuming manual riddling of the fine yeast deposits could be replaced by quickly
5 sinking the alginate beads in the champagne bottle. The disadvantage of such means, in which the beads are not surrounded by a cell-free covering membrane, however, is that they do not show any high mechanical stability, and cannot sufficiently prevent out-growth of especially the relatively smaller microorganisms, whereby the microorganisms can remain in the product after separation of the beads. A multiple use of such medium can only be realized with difficulty.
10 Furthermore, enzymes are usually not immobilizable because of their small size in such alginate beads.

US 4,996,150 describes a process for the microencapsulation of biocatalysts, preferably yeast, as well as their use in the continuous production of ethanol. The biocatalysts are contained in a matrix of an anionic polysaccharide and a cationic polymer. Here, too, the microcapsules do
15 not have any cell-free covering membrane, so that yeast out-growth cannot be sufficiently prevented. Additionally, it is hardly possible to immobilize the smaller biocatalyzers, such as enzymes, with sufficient surety.

US 4,659,662 describes a process for producing alcoholic beverages or bioalcohol by using yeast-containing microcapsules. The yeast can be embedded in a matrix material that is
20 additionally surrounded by a covering membrane, one that, however, is the same as the matrix material. Yeasts immobilized in calcium alginate were named as an example. The covering membrane, however, consists of just a single layer and is, moreover, infiltrated with cells, that is, not cell-free. Thus, out-growth of the yeasts cannot be sufficiently prevented and a sufficient immobilization of enzymes with long-term stability is hardly possible.

25 DE 34 32 923 C2 concerns biocatalysts with immobilized cells that are surrounded by a single-layer, cell-free covering membrane. The covering membrane, which surrounds the interior of the capsule and is not cell permeable, can consist of a ionically or covalently crosslinked gel.

Champagne production is mentioned as an applications example. The covering membrane consists preferably of the same substance as the matrix material within the interior of the capsule. The beads containing a surplus of the crosslinking agent, for example, calcium ions, here out of calcium alginate, are again put into a cell-free substance, here an alginate solution, that forms the covering membrane. The disadvantage with this is that liquefaction of the capsule's contents is not possible without dissolving the covering membrane. Especially with enzymes, however, a liquefied capsule interior facilitates the preservation of natural conformation and thus the enzyme activity. Beyond this, such covering membrane hardly allow for attaining the requirements such as targeted adjustment of permeability and a sufficiently high mechanical stability.

OBJECTS AND SUMMARY OF THE INVENTION

The object of the invention is to make available means for producing and/or treating alcoholic beverages, especially wine or sparkling wine, according to the preamble of the main claim, in which the cells and/or enzymes are permanently immobilized, in which the permeability of the mechanical stability of the covering membrane is adjustable, and in which the contents of the microcapsules can be liquefied.

The invention has the further object of demonstrating applications relating to the means according to the invention.

In accordance with one form of the present invention, a microcapsule for treating a first reactant with one or more further reactants selected from the group consisting of microorganisms and enzymes to yield one or more products includes a core including the one or more further reactants, and a membrane including a first layer that fully encloses the core and a second layer that fully encloses the first layer. The membrane is configured and adapted to be impermeable to the one or more further reactants and is adapted to be permeable to the first reactant and to at least one of the one or more products.

In accordance with another form of the present invention, a method for treating a first reactant with one or more further reactants selected from the group consisting of microorganisms and enzymes to yield one or more products includes at least the step of immersing one or more microcapsules in the first reactant. At least one of the microcapsules includes a core having the

one or more further reactants, and a membrane including a first layer that fully encloses the core and a second layer that fully encloses the first layer. The membrane is configured and adapted to be impermeable to the one or more further reactants and is adapted to be permeable to the first reactant and to at least one of the one or more products.

5 These and other objects, features and advantages of the present invention will be apparent from the following detailed description of illustrative embodiments thereof, which is to be read in connection with the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWING

10 Figure 1 is a simplified schematic diagram of a continuous apparatus for alcohol production constructed in accordance with one form of the present invention.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

15 In accordance with the present invention, the species of microorganisms and/or enzymes used in the production and/or treating of alcoholic beverages, especially wine or sparkling wine, are immobilized in that they are contained in the interior of the capsule, and that a covering membrane completely surrounds the interior of the capsule. The covering membrane for these microorganisms and/or enzymes is not permeable, thus preventing the escape of the microorganisms or enzymes. In order to ensure a substance conversion, the covering membrane
20 for the substances to be converted (eductes) is permeable, also included in this are the nutrients necessary for the microorganisms, for example, glucose; the covering membrane is also permeable to at least a part of the produced or converted substances (products), alcohol and carbon dioxide, for example. The requirements of permeability and mechanical stability are fulfilled by the covering membrane that has at least two layers, radially arranged on top of each
25 other, whereby each layer completely encloses all of the radially arranged layers beneath it. Advantageously the individual layers are bound together ionically and/or covalently.

 Only this multi-layered construction allows the permanent immobilization of cells or enzymes for the production and/or treating of alcoholic beverages, such as wine or sparkling wine. So, for instance, this effectively prevents an outgrowth of yeasts and with this an

accompanying damage of the microcapsules. With the high mechanical stability, the microcapsules can be used in larger bioreactors without squashing or popping the microcapsules. This increased stability also enables the interior of the capsule to be liquefied, without the microcapsules becoming too mechanically instable. Additionally, the permeability of the covering membrane can be selectively adjusted by a suitable selection of at least two layers, which is of decisive importance for immobilizing the enzymes.

Preferably, not only the outer layers, but also the innermost layer of the covering membrane evidence none of the cells or enzymes contained in the inner part of the microcapsules.

Preferably, at least two layers of the covering membrane consist of different substances. Thus, for example, an outer layer (support layer) can consist of a substance that ensures a high level of mechanical stability, while an inner layer (control layer) consists of a substance that enables a selective adjustment of this layer's permeability and with that the covering membrane's permeability.

According to an advantageous embodiment, the cells and/or enzymes contained in the inner part of the microcapsules are embedded in a matrix. This matrix can be constructed from an alginate compound with a polyvalent cation, for example, calcium, strontium, barium, aluminum and/or iron.

According to another advantageous embodiment, the cells and/or enzymes in the interior of the microcapsules are freely movable in a fluid, which is especially advantageous for maintaining the enzyme's natural conformation. With this, the cells and/or enzymes' natural activity is maintained despite being immobilized. If, by means of a crosslinking agent, the microcapsules are produced by the precipitation of beads of a solution containing cells or enzymes, the interior of the capsule can again be liquefied after the covering membrane's layers are fixed. If, for example, the matrix substance in the interior of the capsule consists of calcium alginate, then the polyvalent metallic cation can be exchanged for a monovalent cation, for example, sodium or potassium, in order to again liquefy the interior of the microcapsule. It is thus preferable that at least one layer of the covering membrane consists of a substance that is different from the substance embedding the cells and/or enzymes and constituting the matrix. As

far as known microcapsules are concerned, where the single layer covering membrane consists of the same substance as the matrix in the interior of the capsule, this liquefaction of the interior of the capsule is not possible, since the covering membrane would in this case also dissolve.

5 In the case of an crosslinkable substance using polyvalent cations for example alginate using calcium, a further advantage of exchanging the polyvalent cations for monovalent cations is that polyvalent cations, such as calcium, are as a rule undesirable in wine production. Thus, for example, a matrix of calcium alginate can become liquefied by introducing the microcapsules into an aqueous solution containing sodium citrate, which causes an exchange of calcium ions for sodium ions. These microcapsules, such as those used in wine production for example,
10 preferably will bind polyvalent ions from the substrate to be converted, such as grape juice, which has an advantageous effect. If these microcapsules' matrix again shows a too-high content of polyvalent cations, then the matrix can be regenerated again by treating it with a solution containing monovalent cations.

Microcapsules with a covering membrane out of one layer as well as out of multiple
15 layers are known in the medical field to immobilize cells or enzymes. Thus, Langerhansian cells are enclosed in a microcapsule, with the covering membrane being constructed from a layer consisting of alginate and poly-L-lysine. (F. Lim et al., Science, 210 (1980) 908-910). EP-A-681834 described microcapsules, with the covering membrane being constructed of several layers, for introduction into living organism tissue. The requirements of such microcapsules are
20 tissue tolerance, a low level of immune reaction, and ability to introduce them into living organism tissue.

By optimizing microcapsule construction with a multi-layered covering membrane with a view to the production or treating of alcoholic beverages, suitable microorganisms, such as yeast used in alcohol fermentation or lactic acid bacteria, and/or enzymes, can be immobilized
25 advantageously. Particularly in the case of immobilizing yeast for alcohol fermentation, stability has to be ensured, despite the production of carbon dioxide and a rapid growth of yeast cells. This is achieved through microcapsules with covering membrane that have at least two layers arranged radially above each other.

The means according to the invention has an advantage over the means already known for the production of alcoholic beverages in that the encapsulated cells or enzymes are permanently immobilized and, because of the simple operations process, can be easily measured out and added and can also be simply, quickly, and completely removed from the product. This allows for a specific influence on the individual production steps without impairing product quality. Additionally, microcapsules containing cells or enzymes can be reused, which leads to cost savings where expensive enzymes are used. Further, a good substance exchange, especially the gas exchange in alcohol fermentation, is ensured because of the hollow spaces present between the capsules even where sedimented microcapsules are involved, as well as because of the microcapsules' easy mobility in a fluid substance.

It can be advantageous to make the covering membrane impermeable to those active substances and/or microorganisms situated outside the microcapsule that could impair the activity of the cells and/or enzymes contained in the interior of the capsule. Thus, some species of yeast used in wine production produce toxins that are harmful to other species of microorganisms. By using microcapsules having a covering membrane that is not permeable by such toxins, such microorganisms or enzymes can be used together, for example, in wine production.

According to a preferred embodiment, the microcapsules contain as microorganisms at least one of the yeast species employed in the wine production process.

According to a further preferred embodiment, there is in the microcapsules at least one species of the lactic acid bacteria that are used in biological acid breakdown in wine production.

Processes to deacidify wine by using cells of the *Leuconostoc oenos* species immobilized in calcium alginate are known from US 4,380,552. These beads, however, having no covering membrane, thus not ensuring sufficient stability and an outgrowth of cells.

Advantageously suited as a means for producing or treating alcoholic beverages are microcapsules that contain enzymes such as pectinases, glucanases, β -glucosidases, proteases, and/or glucose-fructose-isomerases used especially in wine production.

It can be advantageous to immobilize cells as well as one or more enzymes in a microcapsule. Firstly, this can make implementation easier, and secondly, this can especially increase productivity if a product of the microorganism or enzyme is further converted by the other microorganisms or enzymes contained in the microcapsule. Thus, species of yeast used in wine production and their treatment substances such as the yeast cell wall preparations and/or glucose-fructose-isomerases can be advantageously immobilized together in a microcapsule. Aside from an increase in activity of the yeasts used, the quantities of, in part, expensive treatment substances to be used can be reduced.

Activity of the immobilized cells or enzyme-increasing substances can also be encapsulated in a microcapsule. It is thus, for example, known that the activity of lactic acid bacteria decreases with an increase in acid content. By including a cation exchanger in a microcapsule containing lactic acid bacteria, the pH-value can be increased at the site of lactic acid bacteria through an exchange of hydronium ions for, for example, potassium ions, thus increasing the activity of lactic acid bacteria. Further examples of activity-increasing substances are vitamins, such as vitamin B₁, or growth-promoting proteins.

Preferably, at least one layer of the covering membrane consists of at least one polymer. Suitable as a polymer is preferably a polyelectrolyte complex that consists of at least one polycation and one polyanion. Suitable polyanions are, for example, polyacrylic acid, polymethacrylic acid, polyvinylsulfonic acid, polyvinylphosphonic acid, alginate acid, cellulose derivatives, especially carboxymethyl cellulose or cellulose-sulfuric acid ester, shellac or shellac components such as aleuritic acid or shellolic acid. Suitable polycations are, for example, polyethylenimine, polydimethyl dialylammonium, poly-L-lysine or chitosan.

The polyanions or polycations preferably have a mean degree of polymerization of above 100, preferably from 100 to 15,000. For the specific adjustment of the covering membrane's permeability, the polymers preferably used for the layer that determines the permeability (regulating layer) are polymers with a narrow distribution of molecular mass, for example, synthetic polyelectrolytic complexes from polyacrylic acid or polymethacrylic acid with polyethylenimine. Thus, the permeability of a small protein having a size of about 60 kD

through a regulating layer of polyethylenimine (PEI) (molecular mass 1,000,000) and polyacrylic acid (PAS) was determined.

Table 1 shows the portion of protein diffused from the microcapsule in connection with the degree of polymerization of PAS after 60 hours of riddling.

Regulating Layer	Molecular Mass of NaPAS	Degree of Polymerization	Permeability After 60 Hours
PEI/PAS	20,000	215	8.4%
PEI/PAS	60,000	645	18.9%
PEI/PAS	170,000	1828	93.1%

5 Table 1. Permeability of the regulating layer for a 60 kD protein in connection with the molecular mass of a polyanion as sodium-polyacrylate (NaPAS).

As seen in Table 1, the permeability of the regulating layer is determined by the polyions, in this case as a polyanion polyacrylic acid, that has a low degree of polymerization, while the counter ions, in this case as the polycation polyethylenimine with a high degree of
10 polymerization, form the framework. Where there is the same counter ion, the polyelectrolytic complex with a poly ion with a high degree of polymerization has a greater strength than those with a poly ion with a low degree of polymerization. As framework-forming counter ions that take up the polyions with a lower degree of polymerization, polyions with a degree of polymerization higher than 50,000 are preferred.

15 To immobilize the enzymes, at least one regulating layer with a degree of polymerization of the polyanion or polycation of 100 to 1,000 is preferred, whereby the framework-forming counter ions, polycation or polyanions have higher degrees of polymerization. To immobilize microorganisms such as yeast, in contrast, higher degrees of polymerization are sufficient; here degrees of polymerization of 1,000 to 15,000 of polyanions or polycations in the regulating
20 layers are preferred, whereby here, too, the framework-forming counter ions, polycations or polyanions, have a higher degree of polymerization. Polyions with high degrees of polymerization have the advantage of greater strength in the respective layer.

For the strength-determining layer (support layer), polyelectrolyte complexes of synthetic polycations or polyanions with high degrees of polymerization of over 10,000 are preferably used. Natural polycations and polyanions such as alginate acid and chitosan or cellulose derivatives can also be used advantageously; with these, a wide molecular mass distribution does not have a disruptive effect.

Table 2 shows examples for substances in the covering membrane layers, whereby each first row lists whether this structure is preferably suited for immobilization of yeast and/or enzymes. The core can have an alginate for immobilization that can be liquefied after the layers have been built. In each column of Table 2, the layer structure from inner to outer is given.

With a larger number of layers, for example 4 or more layers, a still greater covering membrane stability can be achieved.

	Yeasts	Yeasts	Yeasts and/or Enzymes	Yeasts and/or Enzymes	Enzymes	Enzymes
1. Layer	PEI/CMC	PEI/CMC	Chit/PAS	Chit/PAS	PEI/PAS	PEI/PAS
2. Layer	PEI/CMC	PEI/CMC	Chit/CMC	Chit/CMC	PEI/CMC	PEI/CMC
3. Layer		PEI/CMC		Chit/CMC		PEI/Alg

Table 2. Examples of the Covering Membrane's Layer Structure with Immobilized Yeasts and/or Enzymes (Alg = Alginate, Chit = Chitosan, CMC = Carboxymethyl cellulose, PAS = Polyacrylic acid, PEI = Polyethylenimine).

In addition, natural rubber, polystyrene and/or polymethylmethacrylate or a mixture thereof with one or more polyelectrolyte complexes are suitable as polymers for building a covering membrane layer.

The invention also concerns two applications for the means according to the invention. Thus the means can be used in beer production. Here, microcapsules are to be used with cells from one or more yeast species used in beer production. Likewise, the means can be used for producing low-molecular alcohol such as methanol and/or ethanol, whereby microcapsules with yeasts suitable for high-yield alcohol production are used.

To produce and/or treat wine, for example, using the means according to the invention, the microcapsules are to be put into a grape, berry and/or other fruit juice, apple juice, for example, or into a wine. The microcapsules stay in the solution until partial or complete conversion, such as alcohol fermentation, has taken place. Finally, the microcapsules are removed from the solution.

As opposed to already-known means in which the microorganisms or enzymes used have to be filtered off, this means has the decisive advantage of allowing the cells or enzymes used in the production or treatment process to be simply, quickly, and completely removed in the last step of the process. With this, the time that microorganisms or enzymes remain in the solution can be precisely adjusted.

Because of the microcapsules' size, diameters of one-half to a few millimeters are preferred, the microcapsules can be simply and completely removed from the solution. Mechanical processes are appropriate here, for example by using a sieve, or decanting the liquid remaining after prior sedimentation of the microcapsules. In these removal processes, the microcapsules are not destroyed, so that they can be reused if need be after being stored in the interim in a nutritive solution. Especially in the case of expensive enzymes, production costs can be reduced.

Advantageously, at least in the area of microcapsules, the solution is set to just the temperature so that the cells or enzymes show an optimal activity, whereby a possible influence in the product quality is to be considered.

Various microorganisms or enzymes can be used in microcapsules simultaneously and/or time-delayed. In the case of time-delayed usage, the various microcapsules can be successively introduced into the solution and removed together, or the processing steps of introduction, time spent in the solution, and removal are successively run through multiple times with the same solution by using various cells and enzymes immobilized in the microcapsules. The use of different microorganisms or enzymes can thus be used selectively to increase the complexity of, for example, a wine.

A fluidized bed reactor, which contains the means according to the invention, can be used as a bioreactor for production of low-molecular alcohol, especially ethanol, or alcoholic beverages, especially wine or sparkling wine. Suitable reactor types are also listed in Lüders, "Technologie mit immobilisierten Hefen" [Technology with Immobilized Yeasts], Brauwelt 1994, 57.

The bioreactor can also have at least one tube that contains the microcapsules. The two tube openings are advantageously sealed with sieves that retain the microcapsules. Thus a separation of the microcapsules from the converted solution can be omitted. The preferable diameter of suitable tubes is in the area of one or more centimeters.

The liquid to be converted is guided through the tube, whereby numerous tubes can be connected parallel and/or serially to another. In the case of a simultaneous use of various microorganisms or enzymes immobilized in microcapsules, it is preferable that tubes with the same content be joined parallel to each other, whereby groups of tubes with different contents joined parallel to each other are connected together serially.

It can be advantageous to connect together individual tubes serially so that the solution flows through the tubes in succession.

To optimize the activity of the microorganisms or enzymes used, it is advantageous to set the temperature of the inner part of the tubes, that is the microcapsules and their surrounding solution. Here, it is advantageous for a single tube or a group of tubes to have a shared temperature-settable sleeve. The activity of the cells or enzymes can be specifically lowered by cooling the microcapsules enclosed in the tubes.

In an apparatus for the production of low-molecular alcohol, especially ethanol, that has at least one bioreactor, the glucose-containing liquid to be fermented goes from the storage tank into the mixer. There it is mixed with the backflow from the still and then put into the microcapsule-filled bioreactor, where the actual conversion of carbohydrates into alcohol takes place. The alcohol is then separated in the heating vessel and in the distillation column and gathered in the collection tank for the primary product. The separation of alcohol from the remaining liquid occurs by using the different boiling points. The remaining liquid from the still

can, after cooling down in the heat exchanger, again be enriched in the mixer with fresh liquid and go renewed into the bioreactor. In order to avoid an excessive thinning of the glucose in the circulating liquid, a part of the aqueous portion is regularly removed and gathered as a by-product in a collection tank.

5 An apparatus for the production of alcoholic beverages has a greatly simplified structure. The liquid to be fermented is taken from a storage tank and put into an bioreactor according to the invention, where the liquid sits for a period of time or, for example, circulates through a tube reactor. The alcohol-containing product is routed from the bioreactor into a collection tank after the time required for a partial or complete conversion.

10 Further details of the apparatus can be found in the following descriptive section in which, with the drawing, an example of an embodiment is described in more detail. The attached figure (Figure 1) shows the schematic structure of a continuous apparatus for alcohol production. The liquid to be fermented is taken from a storage tank (1) and put into a mixer (2), where it mixes together with a liquid backflowing from the still (4). This mixture is converted
15 into alcohol in the bioreactor (3) from the yeast immobilized in the medium according to the invention. The alcohol-containing fluid is put into a still (4). From there, an alcohol-containing gas phase is routed into a distillation column (5). There, the different boiling points of alcohol in contrast to water are used to enrich the alcohol that is gathered in a collection tank for the primary product (6). The alcohol-lacking portion of the pre-fermented liquid is taken out of the
20 still and cooled in a heat exchanger (8) and routed back into the mixer (2). In order for the liquid in circulation to contain a minimum level of the substances to be converted, especially glucose, a part of the watery portion is regularly removed from the still (4) and gathered as a by-product in a collection tank (7).

Example of Microcapsule Production:

25 5 g sodium alginate (Kelco Co., Hamburg) are dissolved in 700 ml water. Then 70 g of dry yeast was subsequently stirred into this solution (Oenoform, Erbslöh Co., Geisenheim). This suspension was dropped into a 0.6% solution of calcium chloride. After a few minutes of curing time, the beads containing the yeast cells in a calcium-alginate matrix were first washed with water and subsequently with an aqueous 0.05% solution of polyethylenimine (mean molecular

mass 1 mil, Fluka Co.) and was afterwards washed with an aqueous 0.06% solution of carboxymethyl cellulose (mean viscosity, Fluka Co.). Subsequently, the microcapsules thus obtained were washed with water and once again put into the polyethylenimine and carboxymethyl cellulose solution. After being cleaned with water, the microcapsules were stored
5 in water. The microcapsules have a two-layered membrane cartridge, whereby each layer consists of the polyelectrolyte complex of polyethylenimine and carboxymethyl cellulose. Because the calcium alginate beads comprising the cells were produced first and because the layers of covering membrane were subsequently applied, the covering membrane layers showed no yeast cells that could grow out of the microcapsules. The yeast thus immobilized showed the
10 same activity as yeast immobilized in unlayered calcium-alginate beads.

Although illustrative embodiments of the present invention have been described herein with reference to the accompanying drawing, it is to be understood that the invention is not limited to those precise embodiments, and that various other changes and modifications may be effected therein by one skilled in the art without departing from the scope or spirit of the
15 invention.